The University of Chicago

PHOTOSYNTHESIS IN FLASHING LIGHT

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE PHYSICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY
1941

By
SOL W. WELLER

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PHOTOSYNTHESIS IN FLASHING LIGHT

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I. INTRODUCTION

For years there existed in photosynthesis the difficulty that the shape of the continuous light saturation curve speaks for a dark reaction lasting about 1 min., whereas direct measurement of the dark reaction time by the flashing light method (3) gives around 0.01 sec. for the half-period of the dark, or Blackman, reaction. That problem has been explained by Franck and Herzfeld (6) by the assumption that the limiting reaction at light saturation is one in which a catalyst works on a photochemically made substrate which is unstable; all the substrate which cannot be handled by the catalyst is eliminated by back-reactions. We can avoid, in this way, any accumulation of substrate made by a photochemical process and not removed by the catalyst. A further consequence of the instability of the substrate is that a dark pause between illuminations will not permit the catalyst to continue working on accumulated material; by the time catalyst molecules have recovered from working once, any original excess of substrate will have disappeared by back-reactions. With this picture, then, the dark period found by Emerson and Arnold becomes identical with the working, or recovery, time of the catalyst.

The results of Emerson and Arnold also indicated that low temperature and cyanide showed the similar effects of merely prolonging the Blackman

period. That low temperature should have such an effect we can understand; the action of cyanide, however, seemed to put a blot on the picture given above. Emerson and Arnold had observed that, although cyanide increased the period of the dark reaction, if a sufficiently long dark time were permitted between light flashes, the yield per flash (i.e., the yield of oxygen per unit amount of light) in cyanide became identical with that in the unpoisoned case. If, then, saturation is given by the action of a catalyst on an unstable substrate, we can have only that amount of material carried through the photosynthetic process by a short light flash which equals the amount of catalyst available; the less the number of available catalyst molecules, the less the maximum yield per flash. The result of Emerson and Arnold thus implies that the same number of catalyst molecules operate both with and without cyanide. Now cyanide, presumably acting as a catalyst poison, is supposed to remove a portion of the catalyst molecules from active participation. It is hard to see, then, how cyanide could act merely in changing the recovery time of the catalyst while leaving the number of active catalyst molecules constant. Several possibilities of avoiding this difficulty offer themselves. To find out which one actually is realized, it seemed necessary to repeat Emerson and Arnold's measurements and to extend them.

II. EXPERIMENTAL

Two pure strains of the unicellular green alga *Chlorella pyrenoidosa* were used (one obtained originally from the collection of Pringsheim, the other from Emerson); these strains have been grown in this laboratory for several years and exhibit essentially the same behavior. The cells were grown for about 4 days in Knop solution of the following composition:

	grams per liter of solution
$MgSO_4 \cdot 7H_2O$	2.5
KNO_3	1.2
KH_2PO_4	1.2
$Ca(NO_3)_2$	0.5
$FeSO_4 \cdot 7H_2O$	1.7×10^{-4}

Four per cent carbon dioxide in air was bubbled slowly through the culture flasks during the period of growth, and the flasks were placed about 25 cm. above a 100-watt lamp ($I \sim 2000$ lux) during this period Fully developed cultures were kept in the icebox when not in use; in general, a given culture was used for about 2 weeks. In all cases a culture taken from the icebox was given a 15- to 20-min. refreshing period under growing conditions (light; carbon dioxide passing through) before being used in an experiment. On the average, 1 mm.^{3} of the cells could produce about 1 to 1.5 mm.^{3} (at 20°C.) per 5 min. of continuous illumination.

Photosynthesis was measured manometrically in conical or rectangular glass vessels of about 18-cc. capacity, the vessels being attached to Warburg[1] manometers. In the course of an experiment the following procedure was usually observed: after the culture had been refreshed, that volume of cell suspension was taken which would give during the experiment a pressure change of about 0.5 cm. per 5 min. (determined by previous trials). This volume was then centrifuged at a moderate speed for 10 min., and the cells were finally taken up in water or in Warburg's No. 9 carbonate buffer,[2] depending on whether a mixture of 4 per cent carbon dioxide and 96 per cent nitrogen or air was to be used as the vapor phase.

The temperature of the water bath in which the manometric vessels were immersed could be kept constant to within 0.005°C. if, as in most of the measurements, a temperature was chosen in the neighborhood of 20°C. At temperatures differing greatly from room temperature the fluctuations were much larger (~0.1°C).

Flashing light was obtained by the use of a sector rotating before a source[3] of continuous light. Emerson and Arnold preferred to employ a source which itself provided discontinuous lighting (condenser discharges through a neon tube). The advantage of such a procedure is that the duration of the light flashes is very short compared with the dark reaction time, but it has the disadvantage that the light intensity prevailing during the phases of short duration has to be made exceedingly high to insure saturation. This high intensity is difficult to get and to keep constant. Moreover, it is conceivable that the extreme intensity could do some harm to the plants, and, finally, it is impossible with Emerson and Arnold's light source to use the same intensity used for flash illuminations also for a continuous irradiation. For these reasons it was deemed advisable to employ a flash light set-up similar to that of Trelease and Craig (2, 12).

Short light flashes with varying dark times between flashes were obtained by means of a 20-in. sector. The sector was divided into thirty-two radial sections, of which sixteen were removed to a depth of 5 in. from the outer edge of the sector. The sector was driven by a synchronous motor

[1] See reference 14. Even though one molecule of oxygen is produced per molecule of carbon dioxide consumed, it is possible to use a manometric method by virtue of the differing solubilities of carbon dioxide and oxygen in the liquid used in the Warburg cell. If a carbonate buffer is used, of course, the pressure change is due exclusively to the production of oxygen.

[2] This buffer was made up to be 0.085 *M* in potassium bicarbonate and 0.015 *M* in potassium carbonate. The concentration of carbon dioxide in the buffer is 9×10^{-5} *M*.

[3] In most experiments a high-power mercury arc operating on alternating current was used. The intensity of this arc fluctuates periodically with the voltage; that does not constitute an objection to the use of the arc as a continuous source of light for most of the experiments, as will be further discussed below.

at the constant speed of 450 R.P.M. One, 2, 4, 8, or 16 flashes per revolution could be obtained by symmetrically covering up 15, 14, 12, 8, or 0, respectively, of the open radial sections with black paper; each black paper section was just large enough to cover one of the open sections of the sector and was attached to the sector with plasticine.

Some experiments were also done with flashing light of equal light and dark times. For this purpose a two-bladed 12-in. sector was used. This sector was also run by the synchronous motor, and varying lengths of the light and dark periods were obtained through the use of an intermediate gear system.

For the equal light and dark time experiments a 500-watt projection lamp was used as light source. This lamp gave a maximum illumination of ~43,000 lux on the Warburg vessels, the illumination being measured with a Weston photronic cell which had been previously calibrated against a standard lamp.

The light source for the short light flash experiments was a Westinghouse Type A-H6 lamp. This is a 1000-watt, high-pressure, water-cooled mercury arc run on a 1200-volt transformer. The arc permitted an illumination of the algae with about 90,000 lux. To reduce the light intensity a set of neutral screen filters, constructed out of wire gauze and calibrated with a photronic cell, was used. It was necessary to remove the ultraviolet from the light emitted by the arc, since photosynthesis is irreversibly inhibited by ultraviolet light. For this purpose 2 cm. of a quinine sulfate filter containing 3 g. of quinine per liter was used; this removes ultraviolet up to ~3600 Å.

The large sector was situated (with respect to the optical system) in a plane which contained a real image of the quartz capillary of the mercury arc. This image was narrow,—a desirable feature from the standpoint of decreasing the proportion of time spent in transition periods between completely light and completely dark as the sector rotates. The arc runs on 60-cycle A.C.; it is, therefore, extinguished each time the voltage drops below a certain value and starts to burn again as soon as a suitable value of the voltage is reached. Of course, the light emission will not stop entirely the moment the arc is interrupted, but the 240 fluctuations of the intensity of the arc per second must be taken into account. The fluctuations are easy to observe by using the sector, which rotates at a submultiple of 120 cycles, as a stroboscopic disk. In spite of the fluctuations, one can use the illumination of this arc in experiments on photosynthesis as a continuous illumination if the light intensity in the dark phase drops considerably below the saturation value for only an exceedingly short time (*vide infra*). For experiments with light flashes it is necessary to exercise care in adjusting phase relations between rotations of the sector and intensity fluctuations in such a way that a sector opening falls in front of the arc image just during the bright phase of the latter.

The large sector made 7.5 complete revolutions per second; each light flash, therefore, had a duration of $1/32 \times 1/7.5 = 0.0045$ sec. With all but one of the sector openings covered up, the time between flashes (τ) was $0.133 - 0.004 = 0.129$ sec.; with two openings symmetrically placed, $\tau = 0.133/2 - 0.004 = 0.062$ sec., etc.

III. RESULTS AND DISCUSSION

1. Flash experiments at room temperature in absence of inhibitors

Emerson and Arnold's method of determining the dark period with light flashes consists in measurements of the rate of photosynthesis produced by flashing light interrupted by dark periods of varied length. From these observations one calculates the yield per flash, which is plotted against the

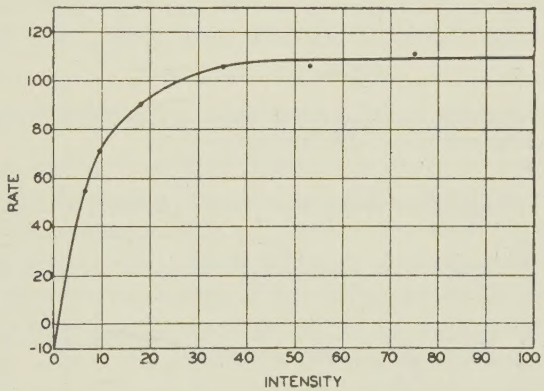

FIG. 1. Saturation curve. 1000-watt mercury arc; No. 9 buffer; 19.6°C.

length of the dark period. The yields per flash were obtained by dividing the observed rate by the number of sector openings used. Since the rate of continuous illumination in our case was measured with the help of the arc, the intensity of which fluctuates, it was necessary to estimate the time for which the intensity drops considerably below saturation. Stroboscopic observations show that the light is sufficiently strong for about four-fifths of the time. The dark pauses last, therefore, $\sim 1/600$ sec. The intensity at the minimum is, according to measurements with calibrated filters, about 5 per cent of the maximum. From saturation curves (see figure 1, for instance) gained with the arc uninterrupted by the sector, one can calculate that even at the minimum the intensity suffices to give ~ 65 per cent of the saturation rate if the arc without filters is used. The correction is, therefore, smaller than the possible error of observation for the full arc illumination. The error will also be small at a value of the integrated intensity of 31,000 lux.

Figure 2 shows a set of typical measurements. The ordinates are the observed rates; the abscissas represent the integrated light intensities as measured with the photronic cell; the parameter of the set of curves is the number of light flashes per second. The curve with the highest rates (marked "continuous") is a saturation curve without interruption by the sector. The most important result which can be read from the set of curves

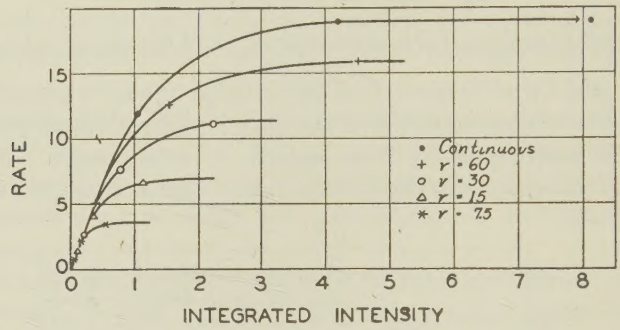

FIG. 2. Saturation curves with flashing light. γ, the number of light flashes per second, is the parameter of the different curves.

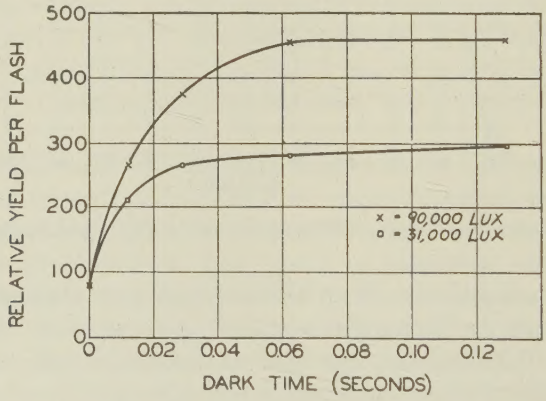

FIG. 3. Yield per flash curves for two different light intensities

is the fact that the saturation values depend upon the number of flashes—i.e., upon the length of the dark period. One gets, therefore, in the range of the higher intensities quite different rates for a given value of the integrated light intensity. In the region of the low intensities, on the other hand, the rate depends only upon the integrated intensity and is independent of the dark pauses. Consequently, it would be futile to draw any conclusions about the duration of the dark reactions while using light flashes whose intensity is not great enough to produce flash saturation. It

is not quite apparent whether Emerson and Arnold fulfilled this condition in their measurements, but since their final result agrees very well with ours, the authors must have used an intensity which was sufficient. The upper curve in figure 3 shows a plot of the yield per flash *versus* the dark time between the flashes. Since the condition of flash saturation was fulfilled for all values of the dark time, the curve can be used for a calculation of the velocity of the dark reaction. The shape of the curve can be very well represented by an exponential function; the dark reaction corresponds, therefore, to a reaction of the first order, in accordance with Arnold's previous observations. The time necessary for the reaction to run half its course is 0.013 sec. at a temperature of 19.6°C. For the lower curve of figure 3 the saturation condition is no longer well fulfilled. Its shape, therefore, depends to a certain degree on the integrated light intensity; although the curve can still be represented by an exponential function with a half-value of 0.009 sec., this value does not have much significance. The general result of the observations described so far is a confirmation of Emerson and Arnold's work.

2. Flash experiments in the presence of cyanide

A repetition of the flashing light experiments in the presence of cyanide gave results which were again in general accordance with those of Emerson and Arnold. The time necessary to complete the dark reaction becomes longer by the addition of this poison, while the yield per flash does not depend upon the presence or absence of cyanide, provided the dark pause is long enough. A more careful measurement of the total curve of yield per flash *versus* dark time in the presence of cyanide reveals, however, the new fact that the curves measured under these conditions cannot any longer be represented by an e function. Consider figure 4, lower curve: for small values of the dark pauses the yield per flash rises strictly linearly, corresponding to the experimental fact that for these dark times the rate in flashing light is the same as that for continuous light. At larger values of the dark pauses the curve bends around, approaching asymptotically the one measured for the non-poisoned algae (compare, for instance, figure 5). It might be mentioned that the magnitude of the effect of cyanide depends somewhat on the culture and the condition of the algae used; in general, an old culture is more susceptible than a young one to cyanide poisoning (i.e., exhibits linearity to greater values of τ. The length for which the second curve is linear increases with the cyanide concentration; by using a sufficiently high concentration of cyanide it was possible to obtain exactly the same photosynthetic rate with flashing light at the slowest frequency (0.13 sec. dark pauses) as was obtained with continuous light.

We have seen that the curves of yield per flash with and without cyanide are qualitatively different. Under normal conditions no linearity is ob-

served even with the shortest time between flashes; with cyanide the linearity can be extended *ad libitum*, at least up to $\tau = 0.13$ sec.

This result, together with experiments on the fluorescence yield in the presence of cyanide as compared with that in its absence (4), led Franck and Herzfeld to the assumption that the catalyst poisoned by cyanide is not the one which is responsible for saturation in the absence of inhibitors, but another one. If the concentration of cyanide becomes great enough to

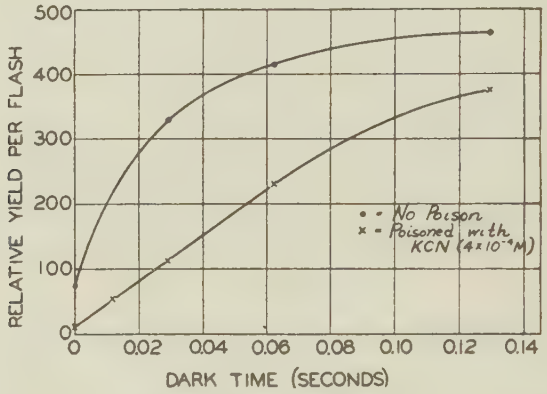

FIG. 4. Yield per flash curves with and without cyanide

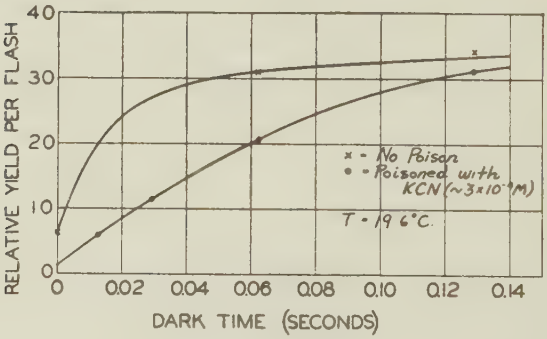

FIG. 5. Yield per flash curves with and without cyanide

reduce the amount of this catalyst below a certain limit, this catalytic dark reaction becomes the limiting one. The plant will be the more sensitive to cyanide the less the velocity of the dark reaction connected with this second catalyst surpasses, in the absence of cyanide, the one responsible for normal saturation.

As is discussed in Franck and Herzfeld's theory, the catalyst limiting in the presence of cyanide (called catalyst A, while the one limiting normally is called catalyst B) is very probably the one observed by Ruben, Kamen,

Hassid, and Devault (13) to be connected with the fixation of carbon dioxide by an acceptor molecule. The shape of the curves presented here fits this assumption very well. Since catalyst A works on a stable substrate, it will, if limiting, follow the course of a reaction of zero order with respect to the substrate, and the constant amount of substrate transformed per unit time will be given by the product of the number of A molecules and the working time for a single transformation.[4]

Even with cyanide present, if we allow a sufficiently long dark time between flashes, A will have transformed enough substrate during the dark pause so that each light flash will photochemically promote enough material to keep busy all the available catalyst B molecules. It is in this region that the situation changes from limitation by A to limitation by B. Increasing the dark intervals (τ) still further means that the limiting reaction becomes more and more the operation of B. The linear portion of the yield per flash curve in cyanide corresponds to limitation by A; the deviation from linearity and the bending over to a horizontal asymptote is related to the transition between limitation by A and limitation by B; and the approach to the same asymptotic value as is reached without poison means that now B is the only limiting factor, as is the case when no poison is present.[5]

3. Flash experiments at different temperatures

A repetition of Emerson and Arnold's experiments on the influence of a lowered temperature in flashing light experiments confirmed the result of these authors that the dark period becomes prolonged. The shape of the curve for yield per flash *versus* dark pause is somewhat changed compared with measurements at normal temperatures; there are some, but only slight, indications that, as in curves measured with cyanide, a reaction of the zero order influences the measurements for small values of τ. The curves in figure 6 give an example of the comparison between yield curves

[4] It is immaterial in these considerations that catalyst A acts before the photochemical steps can take place, while B acts in a reaction which follows the photochemical steps, since the periods of flashes and darkness follow one another periodically.

[5] It might be mentioned here that the work of Craig and Trelease and of Pratt and Trelease on the effect of deuterium oxide creates no conceptual difficulties, as did the first results with cyanide. These workers, using a flashing light set-up similar to that described in this paper, found yield per flash curves for photosynthesis in deuterium oxide like those obtained by Emerson and Arnold under normal conditions but with a prolonged time scale; they concluded that deuterium oxide results in a slower dark reaction but does not affect the photochemical reaction. We can readily understand this on the basis of the mechanism given by Franck and Herzfeld, which provides for catalyst B being involved in the transfer of hydrogen atoms from one molecule to another. Since under normal conditions the reaction involving B is limiting, we expect that the transfer of a deuterium rather than a hydrogen atom can result in a slower over-all rate.

at 20.7°C. and at 4.7°C. The indication mentioned is seen in the fact that at 4.7°C. the absolute rate for flashing of $\tau = 0.012$ sec. was the same within the experimental error as that of continuous light, but already at $\tau = 0.029$ sec. there is a measurable deviation. We expect that at still lower temperatures the proportionality of yield per flash with τ will proceed to higher values of τ; unfortunately, it was not possible to carry out such experiments accurately enough at still lower temperatures. These observations are at least not in disagreement with theoretical expectations. Warburg found that the temperature coefficient of photosynthesis is between 1 and 2 (for a 10° change) at ~15°C., but becomes abnormally high, rising to 4–5, in the neighborhood of 5°C. The conclusion is that probably several catalytic dark reactions contribute at the same time to the limitations responsible

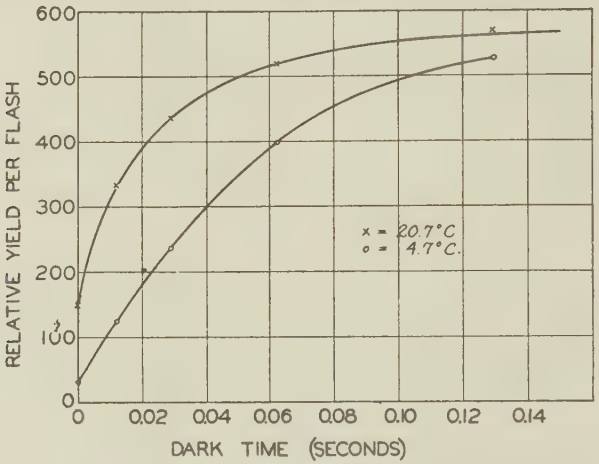

FIG. 6. Yield per flash curves for low and high temperatures

for saturation at these low temperatures. This assumption is, of course, only possible if the dark reaction involving catalyst B, which is responsible for saturation at normal temperature in the absence of inhibitors, has a smaller temperature coefficient than the reaction involving catalyst A and perhaps also a third catalyst called C, the functions of which will be discussed in the next section. Experiments on fluorescence (4) carried out at 1°C. indicate that a limitation by catalyst A does indeed play a decisive rôle at that temperature.

4. Saturation in flash light and continuous light in the presence of hydroxylamine

Since it was possible to differentiate by the method of the flash illumination between limitations produced by catalyst B and those caused by catalyst A, it seemed of interest to study with this method a case in which

a poison is supposed to act specifically on a third catalyst (catalyst C of Franck and Herzfeld), which presumably liberates oxygen from peroxides made by the photochemical processes of photosynthesis. H. Gaffron (9) has made observations which he interprets as indicating that hydroxylamine is such a poison. His arguments are the following: Hydroxylamine is known to poison catalase (which is the catalyst which splits hydrogen peroxide); it also inhibits normal photosynthesis. On the other hand, it does not, as Gaffron found, influence the special kind of photosynthesis, occurring in some plants under anaerobic conditions, in which carbon dioxide is reduced without evolution of oxygen but with an uptake of hydrogen. Since one has reason to believe that the photochemical parts of both types of photosynthesis are the same (5), it would seem that this difference is related to the fate of the peroxide which is formed during photosynthesis. In normal photosynthesis the peroxide splits off oxygen with the help of a catalyst C; in Gaffron's reduced system the peroxide reacts with hydrogen to form water. A specific poisoning of catalyst C by hydroxylamine would then influence only normal photosynthesis.

The poison was used in our experiments in the form of the aqueous solution of its hydrochloride, since it was found that the free base liberated under the alkaline conditions of buffer No. 9 was rapidly decomposed.

Flashing light experiments were first carried out. It was found that hydroxylamine gives a yield per flash curve which, during its entire course, parallels the curve for normal conditions but is displaced to lower values of yield per flash, the amount of displacement depending on the concentration of poison, of course.

To interpret these results one has to know how saturation curves measured in continuous light are influenced by hydroxylamine. Figure 7 gives an example of such measurements. The comparison of the rates *versus* light intensity measured with and without hydroxylamine show that the percentage inhibition is independent of light intensity; the rates at low light intensities are reduced by the same factor as the ones measured at high intensities.

Two interpretations are possible: Either hydroxylamine, against expectation, inhibits photosynthesis as a narcotic rather than as a specific catalytic poison, or the special catalyst, if limiting, has the same influence on low rates of photosynthesis as on high ones, as contrasted with the behavior of other catalysts. Narcotics like phenylurethan, which are supposed to cover unspecifically surfaces in the cell, are known to reduce all rates to the same amount, presumably by putting out of commission a greater or smaller part of the photosynthetic apparatus by the surface covering. By replacing in that way photosensitive substances in contact with chlorophyll by photoinsensitive ones, they also raise the fluorescence yield of the chlorophyll to the same degree for all values of illumination.

Limitations imposed on catalytic reactions, on the other hand, have an influence only in the realm of saturation intensities normally. If we choose the first interpretation, we lose all understanding of Gaffron's results, mentioned above. If, on the other hand, one does not wish to give up the idea that hydroxylamine poisons catalyst C, one has to explain its abnormal influence on the low rates of photosynthesis. It is indeed possible to offer a plausible explanation for the last-mentioned anomaly by comparing it with the one occurring during the induction period. According to Franck, French, and Puck, a limitation of catalyst C at the beginning of an illumination period is responsible for the so-called short induction period of photosynthesis, in spite of the fact that the duration of

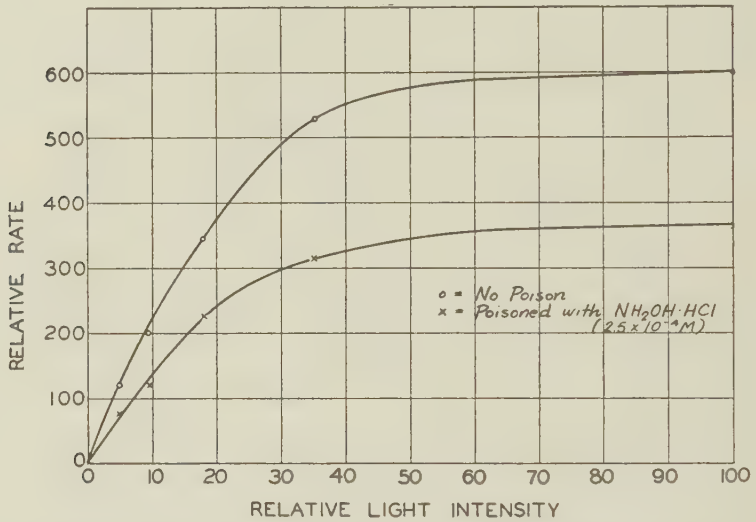

Fig. 7. Continuous light saturation curves with and without hydroxylamine hydrochloride.

the anomaly and the percentage loss of photosynthesis are constant from saturation intensities far down to intensities giving a rate of photosynthesis small compared with the saturation rate. The authors explain many experiments on the anomalies of the photosynthetic rates and of the fluorescence yield during the induction period by the assumption that catalyst C is deactivated by a slow oxidation and reactivated by freshly formed carbohydrates. The ratio between the active and the inactive parts of C becomes approximately proportional to the steady rate of photosynthesis, the induction loss occurring during the time needed for the transition of the ratio to its final value. Once adapted, the concentration of the active part of C is great enough to cause no further limitations, since the total amount of C, the sum of the concentrations of the active and non-active

parts present in the chloroplast, is apparently sufficient. If we apply these ideas of Gaffron (7) to the present problem, the following picture is given: The ratio between active and inactive parts (oxidized and reduced) will not be influenced by hydroxylamine, but the total concentration of available catalyst will be reduced by a sufficient concentration of this poison to such an extent that the active part is deficient for all intensities in the same percentage. More experiments are necessary to confirm the hypothesis offered here, but it may be mentioned that some preliminary experiments on the fluorescence of algae in the presence of hydroxylamine are in favor of the hypothesis.[6]

5. Influence of hydrogen peroxide in the absence of catalase on the rate in flashing and in continuous light

Finally, we may refer briefly to the result of an experiment with hydrogen peroxide. It is known that the poisoning by cyanide of photosynthesis in algae is readily reversible, i.e., the inhibition disappears when the cyanide is washed out and the algae are suspended again in a poison-free medium. It is also known that hydrogen peroxide has a negligible effect on photosynthesis, presumably because it is decomposed by the catalase present in the plant cell before it can do any damage. Gaffron (8), however, found that if small amounts of hydrogen peroxide are added to a suspension of *Scenedesmus* in which the catalase activity has been removed with cyanide, a relatively irreversible inhibition of photosynthesis (not respiration) is observed; this inhibition is observed only at high light intensities. To see if the same effect is present with *Chlorella* and to analyze further the nature of the action of hydrogen peroxide, the following experiment was performed: To a *Chlorella* suspension in 10^{-3} M potassium cyanide was added enough pure, dilute hydrogen peroxide to make the solution $\sim 5 \times 10^{-4}$ M in hydrogen peroxide. After standing for 5 min., the suspension (and a control suspension containing potassium cyanide but no hydrogen peroxide) was centrifuged, washed twice with nutrient solution, and suspended in No. 9 buffer. Illumination with continuous light showed that the saturation rate was reduced to 50 per cent of the rate found in the non-treated algae. On the other hand, flashing light with $\tau = 0.03$ sec. showed no difference between the treated and non-treated algae. We conclude that

[6] The experiments done in coöperation with Dr. French show that the steady state of fluorescence is increased by hydroxylamine at all intensities. However, a difference between the influence of this poison and that of a mere narcotic is seen in the fact that a rise of the fluorescence (for a half minute or more, dependent on the strength of the illumination) is superimposed on the usual anomalies of the induction period. Exactly that is to be expected according to our picture, since the accumulation of peroxides responsible for the rise of the fluorescence takes some time until equilibrium between production and removal by splitting off of oxygen is reached.

hydrogen peroxide (not readily removed by catalase poisoned by potassium cyanide) attacks the molecules of catalyst A. The difference in its behavior from that of cyanide is the irreversible destruction of the A molecules.

6. Illumination with flashes of long duration

As a supplement to the group of experiments made with flashes of constant duration interrupted by dark pauses of varied length, experiments were carried out with flash illumination of equal light and dark times. Such experiments originally made by O. Warburg (14) and repeated by McAlister (10) gave indications that sometimes there occurred a dark period which was much longer than the one measured by the method of Emerson and Arnold. Warburg found, for example, that equal periods of light and dark (saturation light intensity in all cases) of $\tau = 15$ sec. gave a photosynthetic rate 15 per cent greater than one-half the rate in continuous light, whereas if the limiting dark reactions involved have periods of hundreths of seconds, we should observe a rate just one-half of that in continuous light if we illuminate only half the time and allow such long dark pauses between flashes. These experiments were repeated by us under a great variety of conditions; not only were results similar to those of Warburg and McAlister found, but also results which varied from those (depending on the internal condition of the algae) in showing a more rapid decrease to the 50 per cent rate and consistent with the presence of only short Blackman periods. The occurrence of such long dark periods is not astonishing. The conditions are that the individual light flashes have a long duration, and that in the particular cases the saturation rate for continuous light is not only given by the limitation of the dark reaction involving catalyst B but is also influenced by the dark reaction involving catalyst A. That will be the case if the amount of catalyst A available is relatively small. Hydrangea leaves are an example of a plant in which that is regularly the case (as indicated by fluorescence phenomena (4)), but the relative concentrations of A and B also vary in *Chlorella*, as one can infer from the different sensitivity which algae show against cyanide.

As was discussed above, the maximum yield obtainable for a short light flash is influenced only by the limited amount of catalyst B present and is independent of limitations by A. The maximum yield obtainable by a long light flash (lasting some seconds), on the other hand, depends on all limitations which influence the saturation value for continuous illumination. If catalyst A is limiting together with B, the concentration of the compound between acceptor molecule and carbon dioxide will run down during the flash, and long dark pauses are required to raise it again to such an amount that the following flash finds the most favorable conditions. The phenomenon of the long dark periods is, then, a method for observing a limitation by catalyst A just as is the observation of the "pickup phenomenon" of carbon dioxide in the dark (1, 11).

IV. SUMMARY

Photosynthesis in *Chlorella pyrenoidosa* was investigated with the use of light flashes. The main experimental results and conclusions reached are the following:

1. At 20°C. and in the absence of inhibitors the half-time for the limiting dark period is ~ 0.01 sec., which is a confirmation of Emerson and Arnold's results; the dark reaction is identified with the recovery period of catalyst B of Franck and Herzfeld's scheme.

2. Potassium cyanide not only prolongs the duration of the dark period, but also changes the shape of the yield per flash curve. The result indicates that potassium cyanide poisons not the catalyst B usually responsible for saturation, but a second one (catalyst A, which is involved in the dark fixation of carbon dioxide).

3. Low temperatures prolong the dark period. The shape of the yield curves indicates that both catalyst A and catalyst B contribute to the saturation phenomena.

4. Hydroxylamine reduces the rates of photosynthesis in flashing and continuous light by the same percentage. An explanation is based on the assumption that the peroxide-splitting catalyst (C) is poisoned.

5. Hydrogen peroxide (added in the presence of cyanide which is later removed) inhibits photosynthesis irreversibly. Since the inhibition disappears in flashing light, we have to assume that a catalyst (probably catalyst A) is permanently destroyed by peroxide.

6. The apparent long dark period found in equal light and dark time experiments depends on internal conditions and seems to be related to McAlister's "pickup phenomenon" of carbon dioxide.

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